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Ammonia - LCFA synergetic co-inhibition effect in manure-based continuous biomethanation process

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Abstract

In the current study it has been hypothesised that, when organic loading of an anaerobic reactor is increased, the additional cell biomass biosynthesis would capture more ammonia nitrogen and thereby reduce the ammonia toxicity. Therefore, the alleviation of the toxicity of high ammonia levels using lipids (glycerol trioleate-GTO) or carbohydrates (glucose-GLU) as co-substrates in manure-based thermophilic continuous stirred-tank reactors (R_{GTO} and R_{GLU} , respectively) was tested. At $5 \text{ g NH}_4^+\text{-N}\cdot\text{L}^{-1}$, relative methane production of R_{GTO} and R_{GLU} , was 10.5% and 41% compared to the expected uninhibited production, respectively. At the same time control reactor (R_{CTL}), only fed with manure, reached 32.7% compared to the uninhibited basis production. Therefore, it seems that using lipids to counteract the ammonia effect in CSTR reactors creates an “ammonia-LCFA (long chain fatty acids) synergetic co-inhibition” effect. Moreover, co-digestion with glucose in R_{GLU} was more robust to ammonia toxicity compared to R_{CTL} .

Keywords: Ammonia Toxicity; Anaerobic Digestion; Co-digestion; Hydrogenotrophic Methanogens; Lipids.

1 Introduction

Anaerobic digestion (AD) process, is a sustainable technology that is being used widely for organic waste treatment and concurrent bioenergy (i.e. biogas) production (Rajagopal et al., 2013). However, substrates with high nitrogen levels, such as slaughterhouse residues, pig and poultry manure, etc., often result in inhibition of the AD process, due to release of ammonia during their degradation, which affects the stability and performance of the AD process in full-scale reactors (Moestedt et al., 2016). Ammonium ion (NH_4^+) and free ammonia (NH_3) are the two primary forms of inorganic ammonia nitrogen in aqueous solution (Chen et al., 2008). The mechanisms of ammonia inhibition in AD process have been thoroughly studied and NH_3 has been identified as the most toxic form of ammonia. In short, the passive diffusion of hydrophobic ammonia molecules into the microbes' cells may cause potassium deficiency, intracellular pH changes, increase maintenance energy requirements and suppress specific enzyme reactions (Sprott and Patel, 1986). The $\text{NH}_3/\text{NH}_4^+$ equilibrium is affected by the temperature, the pH and the total ammonia ($\text{NH}_4^+ + \text{NH}_3$) concentration in AD process (Rajagopal et al., 2013). In detail, as the pH and/or temperature increase, the conversion of NH_4^+ to NH_3 is consecutively increase enhancing the toxicity effect on the AD process (Angelidaki and Ahring, 1993; Yenigün and Demirel, 2013).

1.1 Conventional strategies to alleviate ammonia inhibition

The suboptimal AD process caused by high ammonia levels results in serious energy production losses for the biogas plants . Up to now, there is not any realistically viable method to alleviate ammonia toxicity in full-scale biogas reactors. Methods such

as dilution of the reactor's content with water or ammonia stripping are expensive or environmentally unsustainable due to increased waste volume (Nielsen and Angelidaki, 2008). The only practical solution is temperature decrease, in order to lower the NH_3 concentrations, but the efficiency of this method is only limited (Kayhanian, 1999). Therefore, the methods used today are either too expensive or time consuming for the full-scale biogas plants, thus the problem of ammonia toxicity on AD process remains.

1.2 Adjustment of C/N ratio of substrate

Another, method that has been proposed is increasing the C/N ratio in the anaerobic reactor (Rajagopal et al., 2013). The C/N ratio is a crucial parameter in AD process, since low C/N ratios could lead to the accumulation of ammonia and result in an inhibition of the anaerobic microbial consortia (Resch et al., 2011). On the other hand, high C/N ratios may cause organic overload of the AD process and VFA accumulation (Nagao et al., 2012). Therefore, optimizing the C/N ratio (between 16/1 and 25/1) in continuous reactors digesting ammonia-rich substrates by co-digesting with other carbon-rich waste, could theoretically alleviate ammonia inhibition (Shanmugam and Horan, 2009). Furthermore, since there is no need for additional equipment, this could be a simple and cost-effective method to counteract ammonia inhibitory effect in full-scale biogas reactors.

Many researchers have tried to improve C/N ratio by co-digestion. For example, Resch (Resch et al., 2011) reported that when animal by-products (mixture of swine blood, rumen content and wastewater with $6.61 \text{ g NH}_4^+\text{-N L}^{-1}$) was co-digested with starch, which improved C/N ratio from 9.7 to 18.3, methane production increased 55% compared to the reactor digesting animal by-products alone. In another study (Kafle et al., 2012), C/N ratio was improved from 2.3 to 7.15 and 12.16 by co-digestion of waste

silage and swine manure ($4.42 \text{ g NH}_3\text{-N L}^{-1}$) under two different ratios (waste silage/swine manure 33/67 and 67/33 % VS basis), led to a biogas production increase of 19% and 40%, respectively compared to only swine manure (Kafle et al., 2012). Rajagopal et al. (2013) reported that increasing C/N ratio of the substrate might reduce total ammonia concentration due to increasing amounts of the microbial biomass (protein generation), if the methanogens present are still active. Therefore, the adjustment of C/N ratio of an ammonia-rich feedstock by co-digestion with carbon-rich substrates is considered a possible method to solve the problem of ammonia toxicity on AD process (Kayhanian, 1999). Contradictory results have however, been reported (Resch et al., 2011) with the addition of corn starch and glycerine in the substrate to improve the C/N ratio of an ammonia stressed CSTR reactor at $6.61 \text{ g NH}_4^+\text{-N L}^{-1}$. Therefore, it is not clear which kind of co-substrates should be co-digested with the ammonia-rich substrates to alleviate ammonia inhibition.

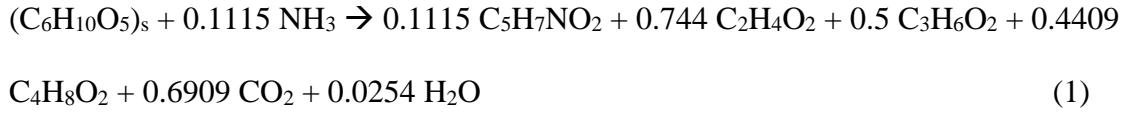
1.3 Microbial biomass generation and ammonium nitrogen fixation by different substrates

When glucose, glycerol trioleate (GTO) and gelatin are used as model carbohydrate, lipid and protein, respectively, different amounts of microbial biomasses are produced per substrate mass. At the same time, different amounts of $\text{NH}_4^+\text{-N}$ are captured by the microbes, from the reactor's liquid phase, due to different amounts of cell biomass ($\text{C}_5\text{H}_7\text{NO}_2$) yields.

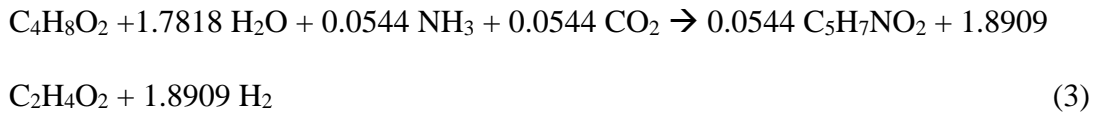
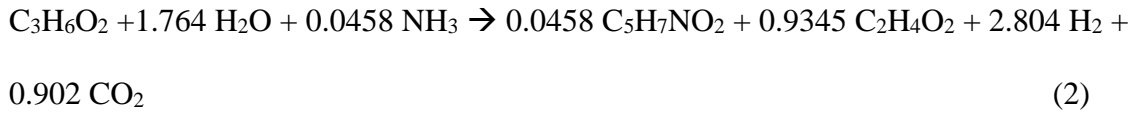
1.3.1 Carbohydrates-Glucose

After soluble glucose is introduced in an anaerobic system, a series of catabolic processes are taking place (Table 1). Specifically, glucose catabolised to acetate

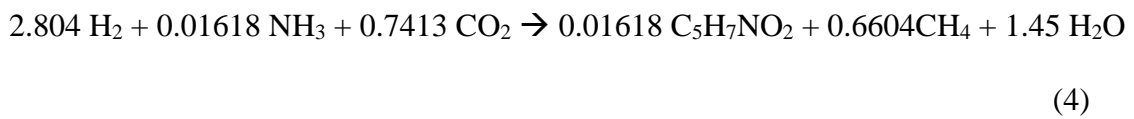
(C₂H₄O₂), propionate (C₃H₆O₂), butyrate (C₄H₈O₂) and cell biomass (C₅H₇NO₂ is the formula of biomass (Angelidaki et al., 1993), which represents a typical amino acid molecule) is produced according the following acidogenic step (Eq. 1) (Hill, 1982):

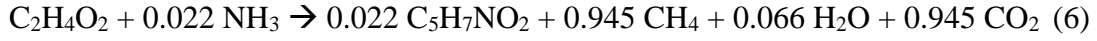
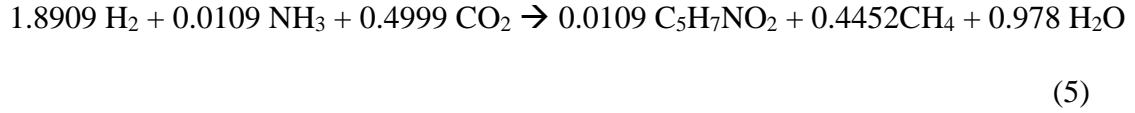


Then propionate and butyrate are degraded to acetate and hydrogen (H₂) in the following acetogenic steps (Eq. 2, 3) (Fedorovich et al., 2003):



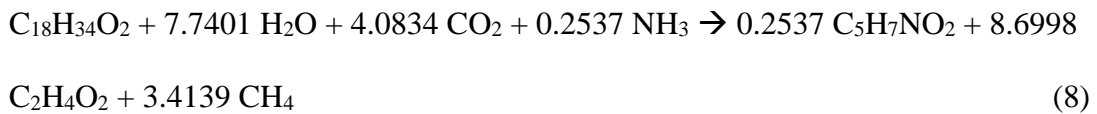
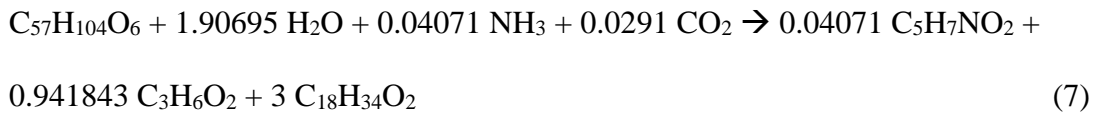
The two pathways to generate methane (CH₄) in the methanogenic step involve a) Hydrogenotrophic methanogenesis (Eq. 4, 5), which are derived from the propionic step (Eq. 2) and the butyrate step (Eq. 3), respectively, and b) aceticlastic methanogenesis, where acetate is broken down to CH₄ and CO₂ (Eq. 6) (Hill, 1982):





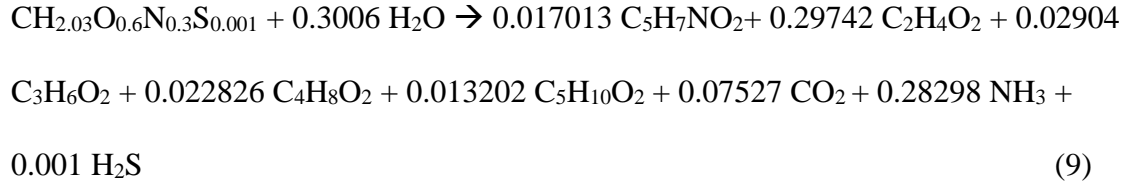
1.3.2 Lipids-GTO

When GTO (e.g. 1 g) is used as substrate in AD process (Table 2), initially degrades to oleate (intermediate product), which consequentially catabolised further by acetogenic bacteria to acetate and H_2 . The stoichiometry of GTO degradation is derived by combining the GTO lipolysis to oleate and glycerol and the glycerol degradation to biomass and propionate. Propionate formation is not accounted because it is assumed to take place instantly as an integral part of the GTO hydrolysis (Schauder and Schink, 1989). The overall GTO-degrading reactions (Eq. 7, 8) are (Angelidaki et al., 1999):

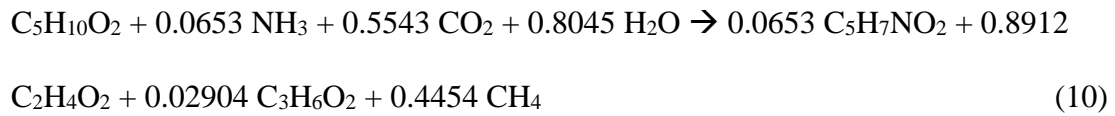


1.3.3 Proteins-Gelatin

Anaerobic digestion, initially catabolises gelatin ($\text{CH}_{2.03}\text{O}_{0.6}\text{N}_{0.3}\text{S}_{0.001}$, Table 3) to VFA according to the following biological reaction (Eq. 9) (Angelidaki et al., 1999):



The main acids formed during gelatin degradation are acetate, propionate, butyrate, and valerate ($\text{C}_5\text{H}_{10}\text{O}_2$). The stoichiometry of the degradation of the intermediates acetate, propionate, and butyrate was adopted from a previous published model and presented in Eq. 10 (Angelidaki et al., 1993; Angelidaki et al., 1999):



Overall, lipids have higher energy content compared to proteins and carbohydrates. Thus, based on the previous calculations, for 1 g of substrate, 23 and 19 mg of $\text{NH}_4^+\text{-N}$ are captured for GTO and glucose, respectively. On contrary, 108 mg of $\text{NH}_4^+\text{-N}$ are released from 1 g of gelatin. Therefore, when 1 g GTO is digested, 4 and 131 mg more $\text{NH}_4^+\text{-N}$ are captured (consecutively reducing the ammonia toxicity effect), compared to glucose and gelatin digestion, respectively. Furthermore, preliminary results derived from modelling simulations have also indicated that the presence of increased lipids concentrations, in reactors digesting ammonia-rich substrates, could theoretically alleviate the ammonia toxicity effect (Angelidaki et al., 1999). Therefore, the hypothesis of this study was that, is possible to use lipids as a co-digestion substrate in order to reduce the high ammonia levels in a continuous reactor and consequently to overcome the ammonia toxicity effect. However, the interaction between lipids and high ammonia

levels during continuous methane production, as well as, the ability of lipids to counteract ammonia inhibition, still need to be tested. Additionally, lipids (long chain fatty acids-LCFA) are reported to inhibit AD process by slowing down the microbial activity and by disorienting essential groups on the cell membranes (Park and Li, 2012).

Therefore, the main aim of the current study was to assess the use of a lipid (i.e. GTO) or soluble carbohydrate (i.e. glucose) as co-substrates in manure-based biogas reactors (continuous stirred tank reactor-CSTR) as a mean to alleviate the toxicity effect of high ammonia levels ($5 \text{ g NH}_4^+ \cdot \text{N} \cdot \text{L}^{-1}$). Both reactors with GTO or glucose as co-substrates were operated at the same conditions. Finally, in order to examine the combined effect of ammonia inhibition and GTO or glucose on the methanogenic populations of the CSTR reactors, the specific methanogenic activity (SMA) of the reactors was tested under $5 \text{ g NH}_4^+ \cdot \text{N} \cdot \text{L}^{-1}$.

2 Materials and methods

2.1 Inoculum and feedstock

The inoculum used in this experiment, was taken from a full-scale thermophilic anaerobic reactor, which was fed with pig and cattle manure (70 to 90% w/w) and organic waste (10 to 30% w/w) in Hashøj Biogas plant (Denmark). Dairy manure was used as feedstock in this study, which was obtained from Hashøj municipality (Denmark). The dairy manure was sieved, in order to remove all the large particles, and avoid blockage of the tubes and then was stored at -18°C . The frozen manure was thawed at 4°C for 2-3 days before fed as substrate in the reactors. The basic characteristics of the manure and inoculum used in this study are shown in Table 4.

2.2 CSTR reactors' experimental setup

Three identical lab-scale thermophilic ($54\pm 1^{\circ}\text{C}$) CSTR reactors were used in this study: R_{GTO} was fed with the mixture of GTO and dairy manure; R_{GLU} was fed with the mixture of glucose and dairy manure and R_{CTL} was a control reactor fed with only dairy manure. The total and working volume of all the reactors were 2.3 and 1.8 L respectively and hydraulic retention time (HRT) was 15 days. Each reactor's setup consisted of a feed vessel, a feeding peristaltic pump, an effluent bottle, two magnetic stirrers for the homogenization of substrate and mixing of the reactor, a water-displacement gas meter and an electric heating jacket unit. At the beginning of the experiment, all three reactors were fed with only manure with an organic loading rate (OLR) of $2.2 \text{ g VS}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$. Then, the OLR of R_{GTO} and R_{GLU} was increased stepwise to 3 and $4 \text{ g VS}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ (mix GTO and glucose with dairy manure as substrate, respectively), while the OLR of R_{CTL} remained the same (data not shown). After all the reactors reached a steady-state (the process was at steady-state when a variation of the methane yield was less than 10% for at least ten consecutive days (Fotidis et al., 2014)), the ammonia concentration of all the reactors was increased stepwise to 4 and $5 \text{ g NH}_4^{+}\cdot\text{N}\cdot\text{L}^{-1}$. NH_4Cl was used as ammonia source. The whole experiment was divided to three distinct experimental periods as shown in Table 5. Considering that the ammonia concentration in CSTR reactors was increased gradually throughout the experiment, the delivery model of the ammonia in the CSTR reactors and the ammonia concentration in the feedstocks are presented in Fig.S1 (Supplementary Material).

2.3 Specific methanogenic activity (SMA) test

The SMAs of R_{GTO} , R_{GLU} and R_{CTL} , after the ammonia concentration reached 5 g $NH_4^+-N \cdot L^{-1}$, were compared as described by (Sørensen and Ahring, 1993). Batch reactors with 118 mL total and 50 mL working volume, respectively were used for the tests. The batch reactors contained 25 mL basic anaerobic (BA) medium (Angelidaki et al., 1990) and 25 mL active biomass retrieved from inside the three CSTR reactors. All the batch reactors (liquid phase and headspace) were flushed with a mixture gas of N_2/CO_2 (80/20% v/v) in order to create anoxic conditions and keep the pH at 7.0 during the experimental period. Subsequently, acetate ($1.18 \text{ g} \cdot L^{-1}$), H_2/CO_2 (2 bar, 80/20% v/v in the headspace) and formate ($3.6 \text{ g} \cdot L^{-1}$), were used as carbon sources. Additionally, reactors only with active biomass were used as blanks to evaluate the residual methane production. All the batch reactors were placed in a shaking incubator at 55°C and all experiments were performed in triplicates (n=3).

2.4 Analyses

Total Kjeldahl nitrogen (TKN), total ammonia, total solids (TS), volatile solids (VS) and pH were measured based on APHA's Standard Methods (APHA, 2005). PHM99 LAB pH meter was used to determine the pH fluctuation in the CSTR reactors. Methane accumulation in the headspace of fed-batch reactors of the SMA test were measured by using Shimadzu-14A gas chromatographer (GC) equipped with a thermal FID detector with hydrogen as a carrier gas (Shimadzu, Kyoto, Japan). The biogas composition in the headspace of the three CSTR reactors was determined by using a gas-chromatograph (GCTCD) equipped with a column of $1.1 \text{ m} \times 3/16$ "Molsieve 137 and $0.7 \text{ m} \times 1/4$ " chromosorb 108 (MGC 82-12, Mikrolab A/S, Denmark), with hydrogen as a carrier

gas . The accumulated volatile fatty acids (VFA) of the three CSTR reactors were measured by using a gas-chromatograph (HP5890 series II) equipped with a flame ionization detector and a FFAP fused silica capillary column, (30 m × 0.53 mm i.d., film thickness 1.5 µm), which uses nitrogen as carrier gas.

2.5 Calculations

2.5.1 Free ammonia

The free ammonia concentrations were calculated from the following equation (Siles et al., 2010):

$$FAN = \frac{TAN}{1 + \frac{10^{-pH}}{K_a}}$$

Where TAN is total ammonia nitrogen, K_a is a dissociation constant, which reflects on temperature, equals to 3.91×10^{-9} for 55 °C and pH is equal to the pH of the reactor's content.

2.5.2 Relative methane production

The methane production of R_{CTL} during P-I was considered as the maximum production yield (uninhibited) of manure in the current study. Moreover, the maximum expected methane production yields of R_{GTO} and R_{GLU} were calculated by summing the production yield of manure during P-I with the theoretical methane production yields of GTO and glucose, respectively. Therefore, relative expected methane production was defined as the percentage (%) of the real methane production yield of each reactor compared to corresponding maximum expected methane production yield.

2.5.3 SMA test

The SMA of the CSTR reactors' active biomass, was calculated as the initial, linear methane accumulation rate in the batch reactors versus time, divided by the active biomasses' VS content in each CSTR reactor (Batstone et al., 2015).

2.5.4 Statistical analysis

All statistical analyses were made using the OriginLab program (OriginLab Corporation, Northampton, Massachusetts). Analysis of variance (one-way ANOVA), for statistically significant difference ($p < 0.05$), was used for methane production and VFA accumulation results of the three CSTR reactors. Student's t-test for statistically significant difference ($p < 0.05$) was used for the SMA results of the different active biomasses derived from the three CSTR reactors. All values presented are the means of independent triplicates ($n=3$) \pm SD.

3 Results and discussion

3.1 Continuous reactors' performance

During P-I, 81.8% and 52.4% relative methane production were achieved for R_{GLU} and R_{GTO} compared to the maximum expected production, respectively before introducing ammonia into all three reactors (Fig. 1a). The results in P-I indicated that a reduced utilisation of the GTO's methane potential has occurred in R_{GTO} before increasing ammonia levels. A previous study (Angelidaki et al., 1990) reported that GTO was found to be inhibitory at 2.5 g L^{-1} in batch experiments and LCFA was considered to be the toxic component of lipid that inhibited the hydrolytic and/or the acetogenic bacteria. Furthermore, the hydrophobic nature of GTO may lead to an inhibitory degradation of microorganisms. Schmidt et al. (2000) reported that, after co-

digested 5% GTO with manure in a CSTR reactor, the methane production decreased and the level of VFA increased overtime compared to digested manure alone. Therefore, these two reasons could explain the incomplete utilization of GTO's during P-I.

In general, methane production of all three reactors decreased alongside increasing ammonia levels. In detail, during P-II the relative methane production of R_{GTO} first kept stable and then decreased at day 70 until a new “inhibited steady-state” (Fotidis et al., 2014) was established at days 82-120 with, 67.1% lower relative methane production compared to the maximum expected production (Fig. 1). Similarly, to R_{GTO} , an inhibited steady-state was also established for R_{GLU} in P-II. In detail, relative methane production of R_{GLU} decreased 41.0% during steady-state (days 60-110), compared to the maximum expected production. On contrary to both R_{GTO} and R_{GLU} , reactor R_{CTL} experienced two methane production decreases. Specifically, two different steady-state levels were established (days 60-80 and days 102-118) with 28.2% and 41.1% lower relative methane production compared to the uninhibited state, respectively. Therefore, it seems that at 4 g $NH_4^+-N \cdot L^{-1}$, the methanogenic consortia in R_{GLU} and R_{CTL} were less inhibited compared to the corresponding consortia in R_{GTO} .

After ammonia concentration in feedstock increased to 5 g $NH_4^+-N \cdot L^{-1}$ (P-III), methane production of R_{GTO} collapsed (89.5% lower compared to the maximum expected production, at day 157) and continue to decline. Reactor R_{CTL} was also affected but was able to establish an inhibited steady-state at day 140 with 67.3% less relative methane production. Methane production of R_{GLU} , also decreased and after day 130 was, in average, 59.0% lower compared to the maximum expected production. At that point, relative methane production of R_{GLU} was significantly ($p < 0.05$) higher compared to both R_{GTO} and R_{CTL} reactors.

During P-II and -III, instead of alleviating ammonia inhibition, as it was hypothesized, GTO led to an almost complete inhibition of the AD process. On the other hand, despite that, they were operating under the same OLR and ammonia levels; R_{GLU} demonstrated a significantly better resistance to ammonia toxicity compared to R_{GTO} throughout P-II and -III. Since the only difference between R_{GTO} and R_{GLU} was the nature of the co-substrate (lipid versus carbohydrate), it seems that the LCFA in the R_{GTO} reactor, combined with the high ammonia levels, created a strong synergistic inhibitory effect with devastating results for the AD process. There are many different inhibitors that could cause synergistic inhibition during AD process (reviewed by Chen et al. (2008)). For example, the concentration of Mg^{2+} could directly affect the inhibition effect of Na^+ . Moreover, many heavy metals, when combined, could also cause synergistic inhibitory effect on the AD process. Finally, the toxicity of ammonia could be enhanced by the presence of increased sulfide levels.

As Lü et al. (2013) have shown, a synergistic inhibitory effect created from high VFA and ammonia levels during AD process was expected for both R_{GTO} and R_{GLU} reactors. However, in the current study, clear indications of a possible “ammonia-LCFA synergetic co-inhibitory effect” was observed. Since R_{GLU} performed significantly better than R_{GTO} , this ammonia-LCFA synergetic co-inhibition seems to affect more the AD process, compared to the VFA-ammonia synergetic inhibitory effect. This can be probably explained because VFA is not a primary inhibitor, and is a result of an inhibited process. Therefore, if the process, escapes inhibition, the VFA would not increase. Moreover, LCFA are apparently stronger inhibitors compared to VFA.

Both ammonia and LCFA individual inhibitory effects are well known (reviewed by (Yenigün and Demirel, 2013; Chen et al., 2014)). Furthermore, previous study indicated

that the presence of LCFA could directly affect the concentration of acetate and propionate during AD process (Angelidaki and Ahring, 1992). However, to date there are only few reports indicating or predicting a potential ammonia-LCFA combined inhibitory effect (e.g. (Astals et al., 2014)). Nevertheless, the exact nature (e.g. inhibition mechanisms), interactions (e.g. the role of VFA in the process) and biochemical parameters (e.g. LCFA and ammonia levels that trigger the process) of this ammonia-LCFA synergetic co-inhibitory effect, merit further investigation.

3.2 VFA Accumulation and pH fluctuation

In general, the total VFA concentrations of all the three CSTR reactors increased alongside increasing ammonia levels (Fig. 2a), which were consistent with the methane production results of the three reactors. Specifically, before increasing ammonia level, the total VFA concentrations of R_{GTO} and R_{GLU} were around $1500 \text{ mg HAc L}^{-1}$ while for R_{CTL} was around $700 \text{ mg HAc L}^{-1}$. The difference of total VFA concentrations among three reactors was caused by the different OLRs. In P-II, the VFA accumulation of all the three CSTR reactors increased and plateaued during the corresponding steady-states of P-II indicating underperforming AD process (Boe et al., 2010). After ammonia concentration of substrate increased to $5 \text{ g NH}_4^+-\text{N}\cdot\text{L}^{-1}$, the concentrations of total VFA kept increasing (Fig. 2a).

Many previous studies showed that $1500 \text{ mg HAc L}^{-1}$ was a threshold of total VFA concentrations for a healthy AD process in both lab-scale and full-scale CSTR reactors (Boe et al., 2010). Before increasing ammonia level, the VFA accumulation of R_{GTO} and R_{GLU} was around the threshold. For R_{GTO} , there was a significant ($p<0.05$) increase of VFA accumulation during P-II which indicated that a serious inhibition occurred. For R_{GLU} and R_{CTL} , an increasing of total VFA concentrations (less than R_{GTO}) led to a

steady-state after increasing ammonia concentration. Therefore, the results of total VFA concentrations further supported the methane production results after the introduction of additional ammonia in R_{GTO} . Moreover, it has been reported before that the degradation of VFA (acetate, propionate and butyrate) in anaerobic reactors was inhibited by low levels of LCFA (Angelidaki and Ahring, 1992) which was in accordance with the VFA results of R_{GTO} .

The pH levels of all the three reactors were around 8.1 before increasing ammonia levels (Fig. 2b). At the end of the experiment, the pH levels reduced to around 7.7 in all reactors. In contrast to the expectations, the pH levels remained in the suitable range for the AD process of all the three reactors (Mata-Alvarez et al., 2000) and were not affected by the high VFA accumulation caused by ammonia inhibition. This could be attributed to the strong buffer capacity of the cow manure, which kept the pH levels from excessive fluctuation (Dias et al., 2014).

3.3 SMA test

Generally, the SMA of R_{GLU} was significantly ($p < 0.05$) higher than the SMAs of R_{GTO} and R_{CTL} (Fig. 3). Moreover, an interesting finding was that the methanogenic cultures in R_{CTL} also had higher SMA than those in R_{GTO} . Specifically, the activity of acetoclastic and hydrogenotrophic methanogens of R_{CTL} was 10.7 and 17.3 $\mu\text{mol CH}_4 \text{ g}^{-1} \text{ VS h}^{-1}$, respectively, which were higher compared to a previous study (SMA were 6.0 and 11.5 $\mu\text{mol CH}_4 \text{ g}^{-1} \text{ VS h}^{-1}$ for acetate and H_2/CO_2 , respectively, under ammonia level of 6 g $\text{NH}_4^+ - \text{N} \cdot \text{L}^{-1}$) (Angelidaki and Ahring, 1993). For hydrogenotrophic methanogens, the SMA of R_{GLU} was 32.6% higher than R_{GTO} . For acetoclastic methanogens and formate-utilization methanogens, the differences of SMA between R_{GLU} and R_{GTO} become more significant (R_{GLU} were 69% and 68% higher, compared

with R_{GTO}). Additionally, when acetate and formate were used as substrate, the SMA of R_{GTO} was even 46.7% and 59.4% lower than R_{CTL} .

Aceticlastic methanogens in all three reactors had the lowest SMA compared with hydrogenotrophic methanogens and formate-utilization methanogens. On the other hand, the SMA of hydrogenotrophic methanogens in R_{GTO} was 62.3% and 51.7% higher than aceticlastic methanogens and formate-utilizing methanogens, respectively. The results of SMA test were consistent with many previous studies which indicated that hydrogenotrophic methanogens are more robust to ammonia inhibition compared with aceticlastic methanogens (Yenigün and Demirel, 2013).

Moreover, the SMA results showed that the hydrogenotrophic methanogens were more robust to ammonia-LCFA synergistic inhibitory effect, compared to aceticlastic and formate utilizing (some hydrogenotrophic (Rea et al., 2007)) methanogens. This ability of the hydrogenotrophic methanogens has been reported in similar (Wang et al., 2015), and also in other toxic anaerobic environments (Symsaris et al., 2015) and underlines the significant role that these methanogens have in the AD process.

4 Conclusions

The results of the current study did not support the hypothesis that using lipids as a co-digestion substrate is possible to overcome the ammonia toxicity effect on anaerobic digestion process. On contrary, they indicated that the usage of a lipid (i.e. GTO) as co-substrate in a manure-based reactor that suffers from ammonia toxicity creates an ammonia-LCFA synergetic co-inhibitory effect with immediate and severe effects on the reactor's performance. On the other hand, when a simple carbohydrate (i.e. glucose)

was used as co-substrates, the reactor was more robust to ammonia toxicity effect compared to the reactor that fed with GTO.

5 Acknowledgements

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Table 1. Calculation of microbial biomass generation and ammonium nitrogen fixation with glucose as AD substrate. Unit: mg compound g⁻¹ glucose; “-” denotes the consumption of the compound during the degradation steps.

	Glucose	NH ₃ -N	HAc ^a	HPr ^b	HBut ^c	Microbial biomass
Glucose	-1000	-10	276	228	240	78
HPr		-3	173	-228		22
HBut		-3	309		-240	20
HAc		-4	-757			31
Overall	-1000	-19	0	0	0	151

^a Acetate; ^b Propionate; ^c Butyrate

Table 2. Calculation of microbial biomass generation and ammonium nitrogen fixation with GTO as AD substrate. Unit: mg compound g⁻¹ GTO; “-”denotes the consumption of the compound during the degradation steps.

	GTO	LCFA	NH ₃ -N	HAc	HPr	Microbial biomass
GTO	-1000	957	-1		79	5
LCFA		-957	-12	1771	0	97
HPr			-1	60	-79	8
HAc			-9	-1831		76
Overall	-1000	0	-23	0	0	186

Table 3. Calculation of microbial biomass generation and ammonium nitrogen fixation with gelatin as AD substrate. Unit: mg compound g⁻¹ gelatin; “-”denotes the consumption of the compound during the degradation steps.

	Gelatin	P _{in} ^a	Amino acids	NH ₃ -N	HAc	HPr	HBut	HVal ^b	Microbial biomass
Gelatin	-1000	200	800						
Amino acids			-800	114	512	65	58	39	55
HVal					20	28		-39	3
HBut				-1	74	0	-58		5
HPr				-1	71	-93			9
HAc				-4	-678				28
Overall	-1000	200	0	108	0	0	0	0	100

^a Insoluble Protein; ^b Valerate

Table 4. Characteristics of the inoculum and the dairy manure used as substrate

Parameter (unit)	Inoculum value \pm SD	Substrate value \pm SD
density (g·L ⁻¹)	1003.52 \pm 1.10	1002.89 \pm 0.78
TS (g·L ⁻¹)	34.85 \pm 0.08	86.9 \pm 0.00
VS (g·L ⁻¹)	22.42 \pm 0.04	63.3 \pm 0.01
Total Kjeldahl nitrogen (g N L ⁻¹)	4.29 \pm 0.24	3.48 \pm 0.13
ammonia (g NH ₄ ⁺ -N·L ⁻¹)	3.22 \pm 0.12	2.1 \pm 0.08
total VFA (mg L ⁻¹)	3286.7 \pm 169.70	5606.4 \pm 195.10
pH	7.82	8.06

Table 5. Operational parameters in the different experimental periods of the three CSTR reactors.

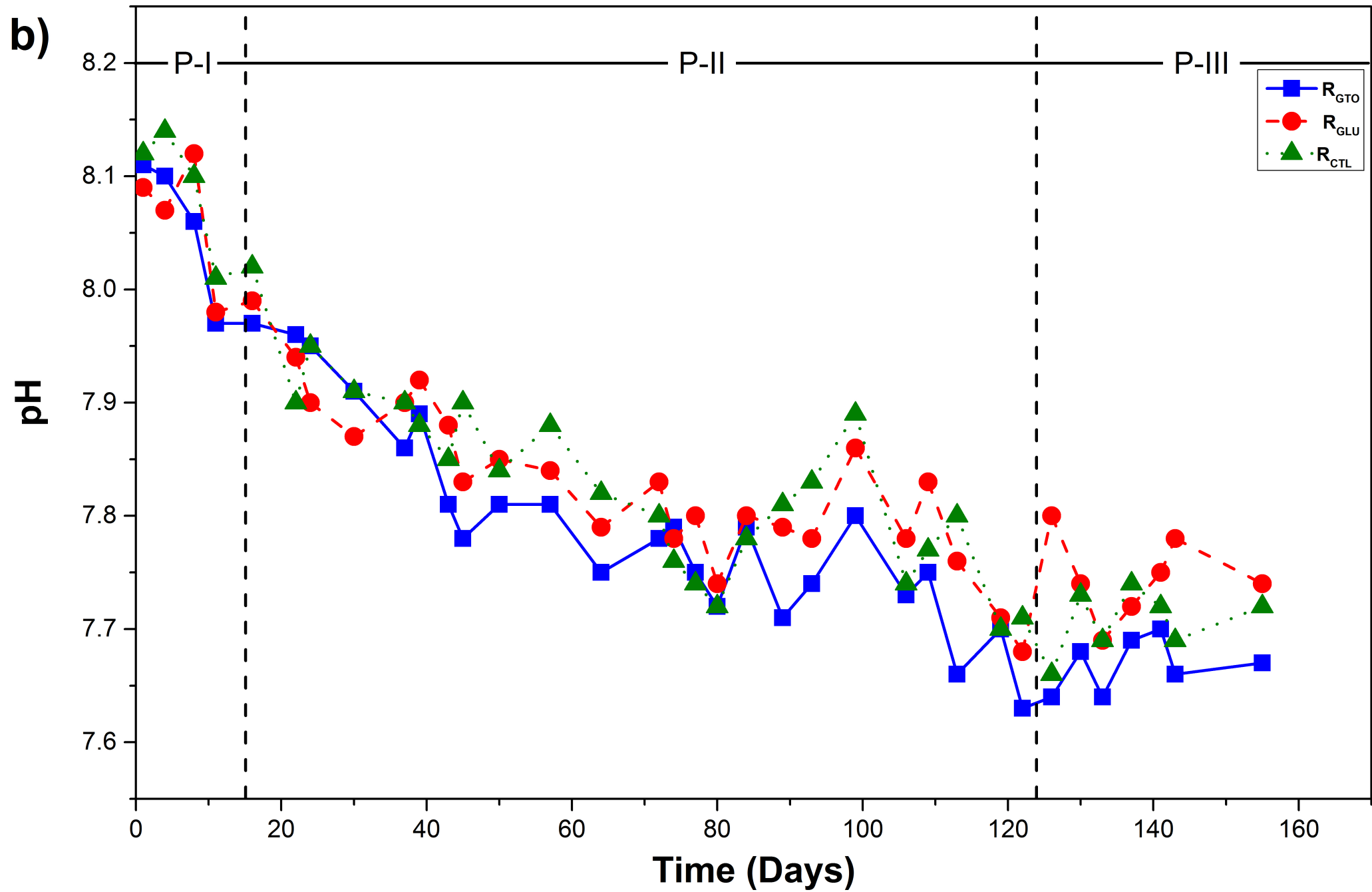
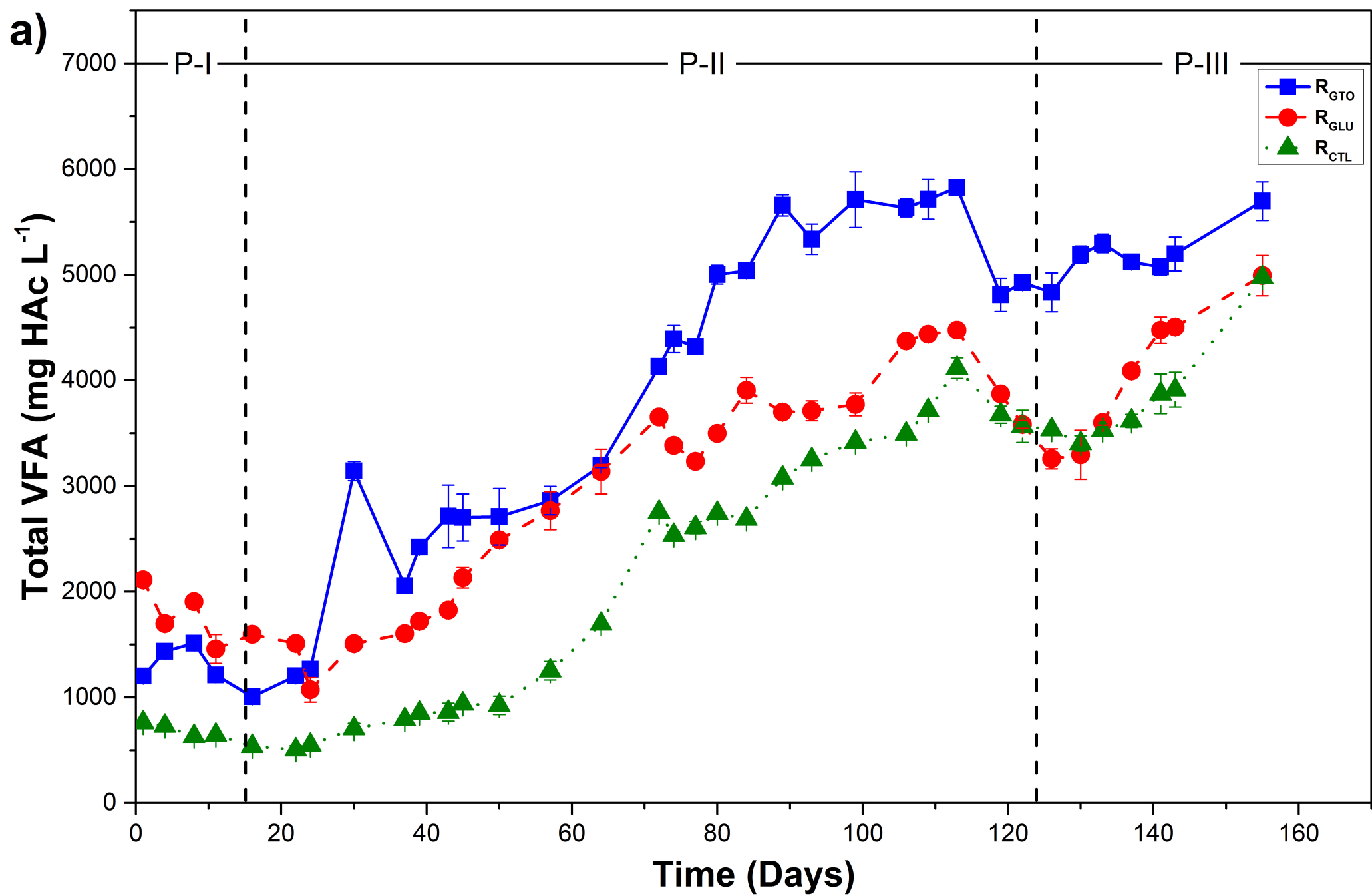
Experimental period (Days)	Ammonia concentration (g NH ₄ ⁺ -N·L ⁻¹)	OLR (g VS·L ⁻¹ ·d ⁻¹)	
		R _{GTO} and R _{GLU}	R _{CTL}
P-I (days 1-15)	2.1	4	2.2
P-II (days 16-123)	4	4	2.2
P-III (days 124-157)	5	4	2.2

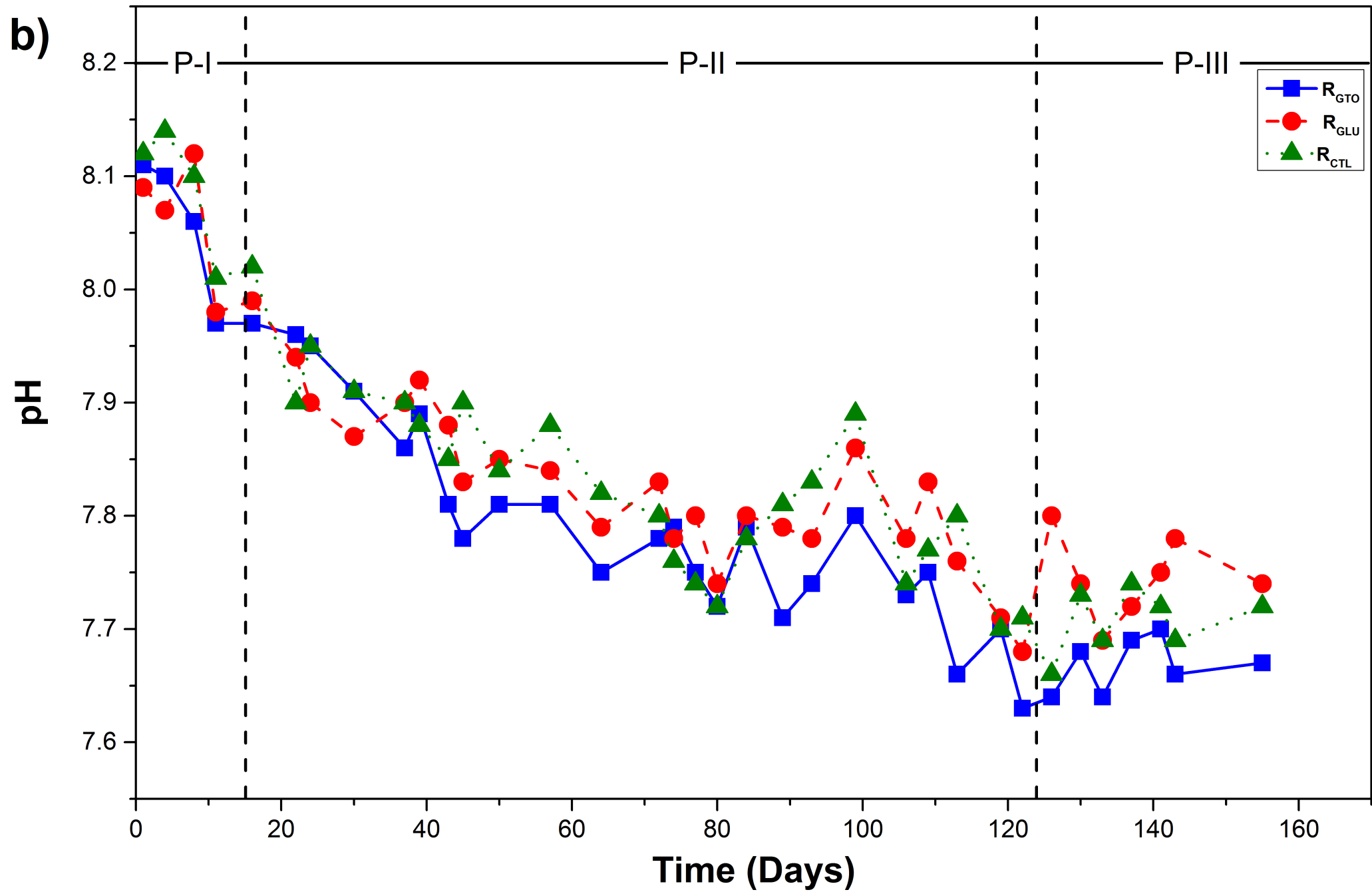
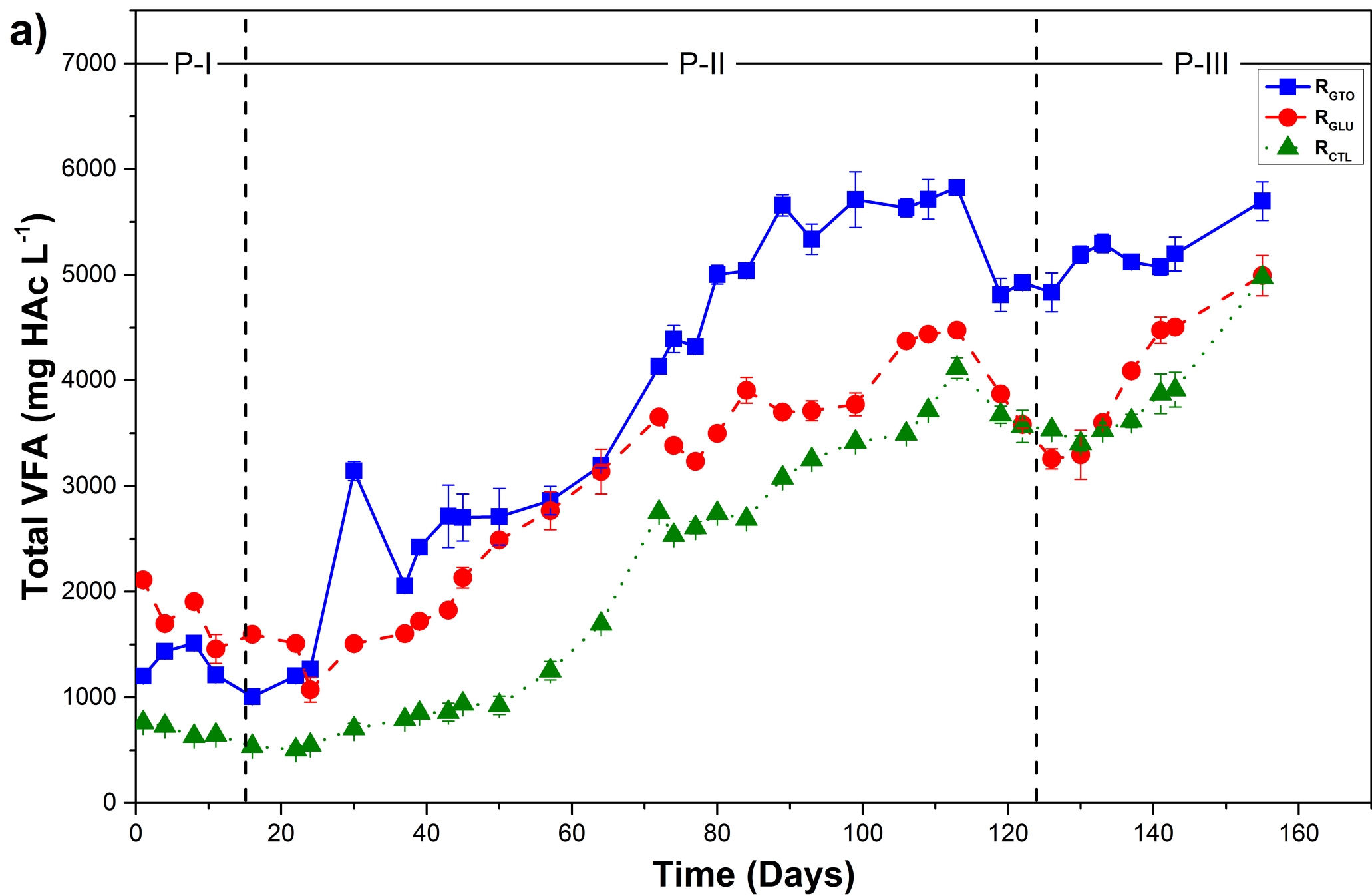
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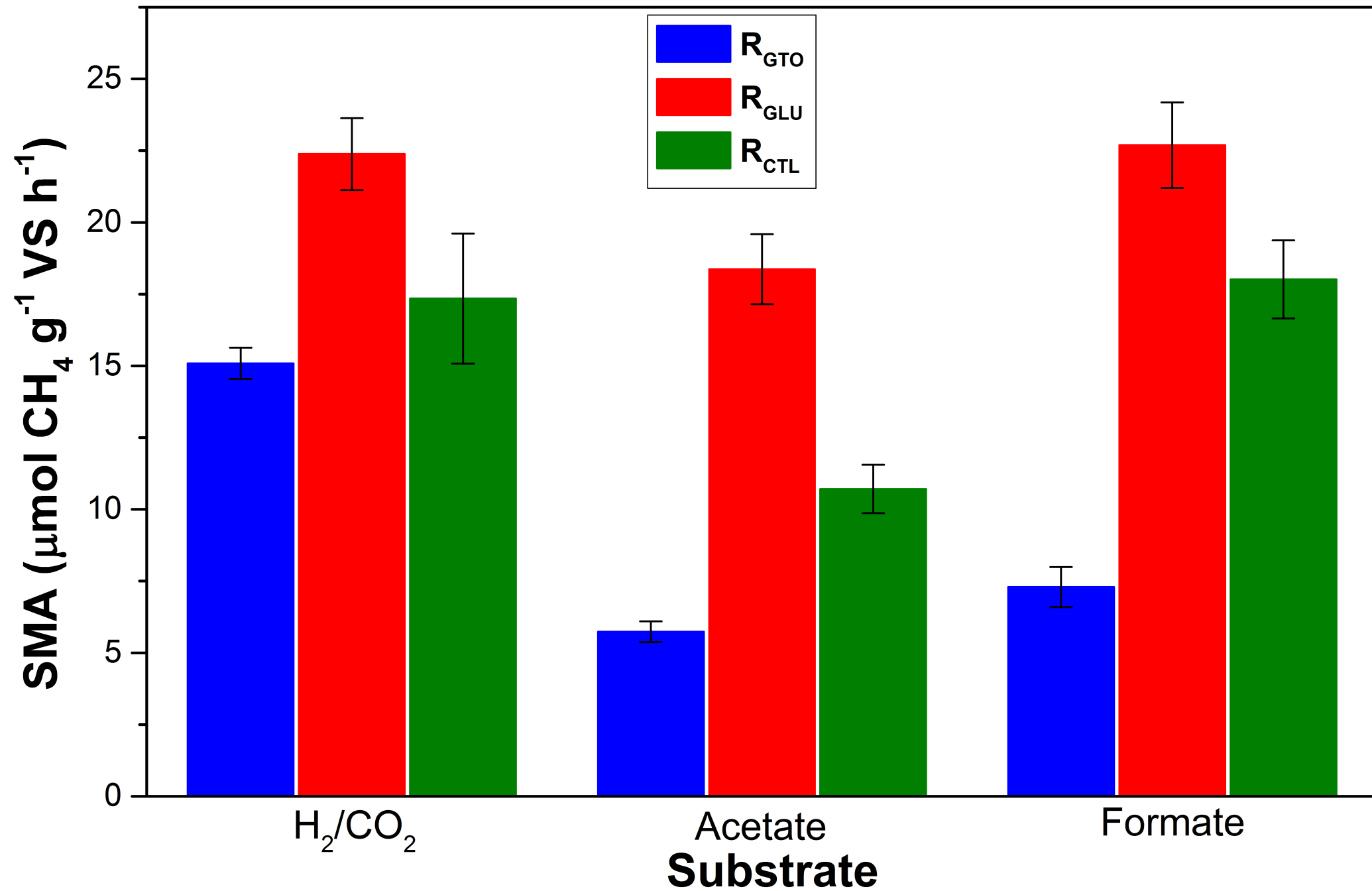
Fig. 1. Relative methane production (a) and methane production rate (b) of the three CSTR reactors.

Fig. 2. VFA accumulation and (a) pH fluctuation (b) of the three CSTR reactors.

Fig. 3. Specific methanogenic activity (SMA) measured for the three CSTR reactors.







Supplementary Material

Ammonia - LCFA synergetic co-inhibition effect in manure-based continuous biomethanation process

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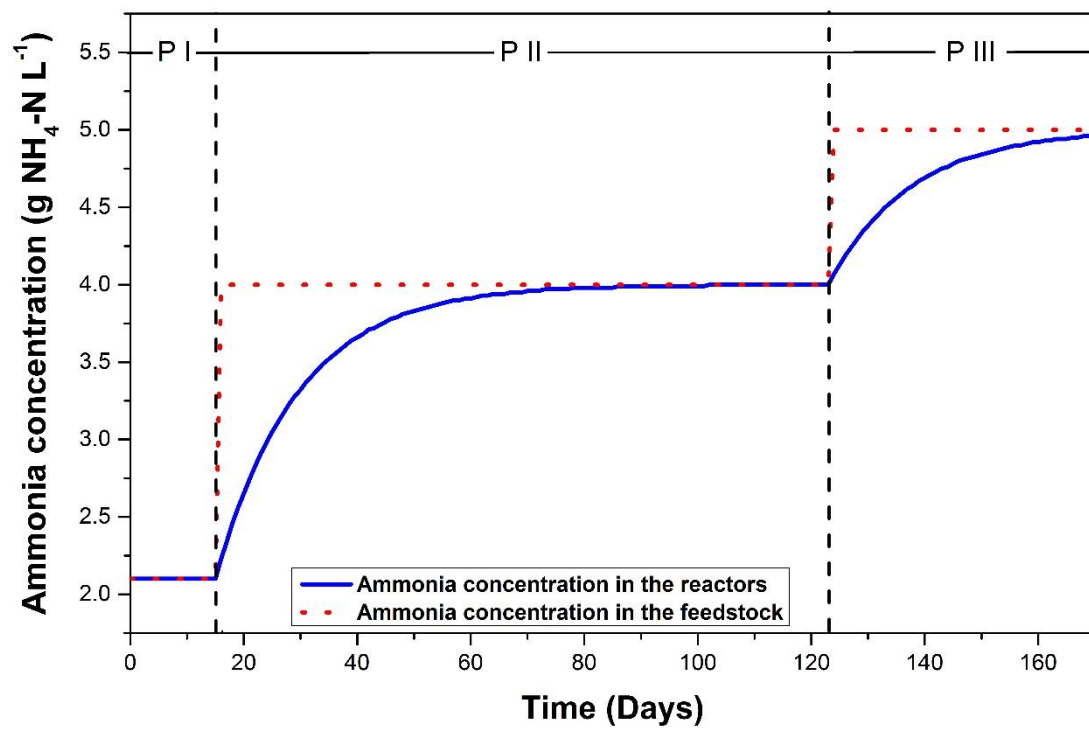


Fig. S1. The delivery model of the ammonia in the CSTR reactors and the ammonia concentration in the feedstocks throughout the experiment